REMARKS

Status of the Claims.

Claims 1-12, and 14-17 are pending with entry of this amendment, claims 13 and 18-32 being previously cancelled and no claims being added herein. Claim 1 is amended herein. The amendment finds support at page 18, lines 11-12.

Objection to the Title.

The Examiner maintained here objection to the specification stating that there is no evidence of a change in the Title. Applicants note that in the Amendment filed on November 4, 2004 (page 2) Applicants changed the Title of the invention from "AMPLIFICATION OF CYP24 AND USES THEREOF" to -- CYP24 EXPRESSION LEVEL AS A MARKER FOR PREDISPOSITION TO CANCER--. Nevertheless for further clarity, the title is amended herein (see amendment to the specification) to recite --**DETECTING** CYP24 EXPRESSION LEVEL AS A MARKER FOR PREDISPOSITION TO CANCER-- thereby obviating this objection.

Objection to the Specification.

The Examiner objected to the reference to GenBank accession numbers U60669 and S78775 in claim 1 alleging that this is an improper incorporation by reference of essential subject matter. Claim 1 is amended herein to eliminate reference to the Accession numbers thereby obviating this objection.

Applicants further note that the referenced GenBank accession numbers are not essential subject matter (see discussion below).

35 U.S.C. §112, first paragraph, new matter rejection.

The Examiner rejected claim 15 under 35 U.S.C. §112, first paragraph, alleging that inclusion of the recitation ". . . at the 95 percent or greater confidence level" in claim 15 presents new matter. Applicants traverse.

At page 42, lines 7-11, the specification states:

> In a particularly preferred embodiment, **the assay is deemed to show a positive result** (*e.g.*, "a prognostically significant level") when the difference

> between sample and "control" is statistically significant (*e.g.* at the 85% or greater, preferably at the 90% or greater, **more preferably at the 95% or greater** and most preferably at the 98% or greater **confidence level**).

The specification thus provides express support for claim 15 and the rejection on these grounds should be withdrawn.

35 U.S.C. §112, first paragraph, enablement rejection.

Claims 1-12 and 14-27 were rejected under 35 U.S.C. §112, first paragraph. The Examiner alleged that the claims contain subject matter (the GenBank Accession Numbers) that was improperly incorporated and that failing such incorporation, the specification does not enable any person skilled in the art to make and sue the invention commensurate in scope with the claims. Applicants traverse.

The Examiner is reminded that M.P.E.P. §2164.01 (Test of Enablement) expressly states that:

> **A patent need not teach, and preferably omits, what is well known in the art**. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984). Determining enablement is a question of law based on underlying factual findings. *In re Vaeck*, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991); *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984).

The Examiner is reminded that the pending claims are not directed to the CYP-24 gene, but to diagnostic methods that involve detection of the previously known CYP-24 gene or gene product:

> . . . (ii) detecting the level of CYP24 nucleic acid or CYP24 protein, within said biological sample, wherein said CYP24 nucleic acid or CYP24 protein is a nucleic acid or protein encoded by **the vitamin D 24 hydroxylase (*CYP24*) gene**; and . . .

The reference to the vitamin D 24 hydroxylase (*CYP24*) gene is not indefinite and methods of detecting the level of a CYP24 nucleic acid or a CYP24 protein are fully enabled. The vitamin D 24 hydroxylase (*CYP24*) gene was **well known** to those of skill in the art as of the April 02, 1999 filing date of the present application as were **methods of detecting expression products** of this gene. Thus, for example:

1) Jones *et al.* (1999) Expression and Activity of Vitamin D-Metabolizing Cytochrome P450s (CYP1α and CYP24) in Human Nonsmall Cell Lung Carcinomas, *Endocrinology*, 140(7): 3303-3310 (Attached as Exhibit A) **illustrates detection of CYP24 mRNA** using a Northern analysis.

2) Kerry *et al.* (1996) Transcriptional Synergism Between Vitamin D-responsive Elements in the Rat 25-Hydroxyvitamin D_3 24-Hydroxylase (*CYP24*) Promoter, *J. Biol. Chem.*, 271(47): 29715-29721 (Attached as Exhibit B) illustrates methods of detecting **induction of transcription of the CYP24 gene**;

In addition, the specification teaches a number of methods of detecting the CYP-24 gene or protein product. Thus, for example:

1) Example 1 of the present application teaches and illustrates the use of **RT-PCR to detect CYP-24 expression levels and provides PCR primers** to perform such RT-PCR.

2) Example 2 of the present application teaches **CYP-24 expression analysis using multi-color fluorescent in situ hybridization (mRNA-FISH)** on tissue sections.

3) Example 3 illustrates measurement of the **expression of CYP-24 at both the transcript and protein levels**.

There is simply no question that the present specification teaches one of skill in the art how to perform the claimed method without undue experimentation. Accordingly, the rejection under 35 U.S.C. §112, first paragraph, should be withdrawn.

Applicants note that they previously amended claim 1 to recite the GenBank accession numbers simply to expedite prosecution. The reference to the GenBank numbers in the specification **was not an improper incorporation by reference, but merely an illustration that the CYP24 gene and protein was well known to those of skill in the art.**

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested. Should the Examiner seek to maintain the rejections, Applicants request a telephone interview with the Examiner and the Examiner's supervisor.

If a telephone conference would expedite prosecution of this application, the Examiner is invited to telephone the undersigned at (510) 769-3513.

QUINE INTELLECTUAL PROPERTY LAW
GROUP, P.C.
P.O. BOX 458
Alameda, CA 94501
Tel: 510 337-7871
Fax: 510 337-7877

Respectfully submitted,

Tom Hunter
Reg. No: 38,498

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Expression and Activity of Vitamin D-Metabolizing Cytochrome P450s (CYP1α and CYP24) in Human Nonsmall Cell Lung Carcinomas*

GLENVILLE JONES, HEATHER RAMSHAW, ANQI ZHANG, ROBERT COOK, VALARIE BYFORD, JAY WHITE, AND MARTIN PETKOVICH

Cytochroma, Inc. (G.J., H.R., A.Z., R.C., J.W., M.P.), Bioscience Complex, Kingston, Ontario, Canada K7L3N6; the Departments of Biochemistry (G.J., V.B., M.P.) and Medicine (G.J.); and Pathology and Cancer Research Laboratories (M.P.), Queen's University, Kingston, Ontario, Canada K7L 3N6

ABSTRACT

Extrarenal 25-hydroxyvitamin D_3-1α-hydroxylase is believed to play a major role in the pathogenesis of hypercalcemia associated with various types of granulomatous and lymphoproliferative diseases and certain solid tumors. In this paper, we describe the cloning of the cytochrome P450 component of the extrarenal enzyme from a human nonsmall cell lung carcinoma, SW 900. The cytochrome P450 for the extrarenal 1α-hydroxylase has an amino acid sequence identical to that of the cytochrome P450 component of the CYP1α, the renal form of the enzyme, and appears to be a product of the same gene. CYP1α messenger RNA (mRNA) and 1α-hydroxylase enzyme activity were detected in two (SW 900, SK-Luci-6) of a series of five nonsmall cell lung carcinoma cell lines. All five lung cell lines were cultured with the same medium under the same conditions, but only two of the five expressed 1α-hydroxylase enzyme; two others (WT-E, Calu-1) expressed high levels of the reciprocally regulated enzyme, 25-hydroxyvitamin D_3-24-hydroxylase, with its specific cytochrome P450 component, CYP24. Although under basal conditions the lung cell line SW 900 expressed only CYP1α and showed 1α-hydroxylase enzyme activity, when treated with small concentrations of 1α,25-dihydroxyvitamin D_3 or high concentrations of 25-hydroxyvitamin D_3, it began to express CYP24 and exhibit 24-hydroxylase enzyme activity. Somewhat surprisingly, SW 900 cells still had detectable CYP1α mRNA some 24 h after vitamin D treatment despite the fact that 1α-hydroxylase enzyme activity was unmeasurable. These data are consistent with the emerging hypothesis that vitamin D through its active form does not directly turn off CYP1α mRNA production but, rather, strongly stimulates CYP24, thereby masking CYP1α activity. The factor(s) responsible for the basal expression of CYP1α in SW 900 and SK-Luci-6 is currently unknown. (*Endocrinology* **140: 3303–3310, 1999**)

THE ENZYME 25-hydroxyvitamin D_3-1α-hydroxylase (1α-hydroxylase) plays a central role in calcium homeostasis (1), and elucidation of the details of its structure and hormonal regulation are expected to provide a wealth of information (2). The enzyme catalyzes the conversion of 25-hydroxyvitamin D_3 ($25OHD_3$) to the hormone 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$], which regulates calcium and phosphate transport in intestine, bone, and kidney. For at least a decade after its discovery, it was believed that the 1α-hydroxylase was exclusively expressed in the kidney (3), and this was further fueled by the knowledge that human patients with chronic renal failure suffer from avitaminosis D, which results in renal osteodystrophy (4). Only in the mid-1980s did it become evident that extrarenal cells (*e.g.* bone, alveolar macrophage, placenta, and keratinocyte) could express the 1α-hydroxylase enzyme activity *in vitro* (5–8). More recently, Mawer *et al.* (9), studying a panel of 16 lung cancer cells, showed that one cell line (NCI H82) exhibited measurable 1α-hydroxylase activity when cultured *in vitro*. In all reports of extrarenal enzyme activity, the 1α-hydroxylase was not up-regulated by PTH and appeared to be poorly down-regulated, just the opposite of the renal enzyme, for which tight regulation by plasma calcium and vitamin D is one of the hallmarks (10). Consequently, unlike the renal 1α-hydroxylase, the extrarenal 1α-hydroxylase is not inversely correlated with the vitamin D-inactivating enzyme activity, $25\text{-}OHD_3$-24-hydroxylase (24-hydroxylase) which converts $25OHD_3$ to the degradation product 24,25-$(OH)_2D_3$ (11). Accordingly, there has been strong evidence presented that the loosely regulated extrarenal 1α-hydroxylase correlates with the appearance of hypercalcemic episodes and thus might be the cause of the hypercalcemia associated with sarcoidosis, lymphoma, and perhaps even some types of solid tumors (9, 12, 13). Underlying the findings to date was always the possibility that the extrarenal 1α-hydroxylase might be the product of a gene different from that coding for the renal enzyme and therefore regulated differently by the calcium homeostatic machinery.

The renal 1α-hydroxylase enzyme is the result of a combination of the activities of three proteins, a specific cytochrome P450 and two general proteins, ferredoxin and ferredoxin reductase. Partially purified preparations of the three proteins have been reconstituted to give the 1α-hydroxylase enzyme activity *in vitro* (14). Very recently, the specific cytochrome P450 (CYP1α), representing the key protein of the renal 1α-hydroxylase enzyme complex has been cloned from rat (15) and subsequently from mouse and human (16–19). *In vitro* transfection studies of CYP1α together with chro-

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Address all correspondence and requests for reprints to: Dr. Glenville Jones, Department of Biochemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6. E-mail: gj1@post.queensu.ca.
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mosomal analyses have indicated that this is indeed the gene responsible for the renal production of $1\alpha,25(OH)_2D_3$ and is defective in the hereditary form of rickets known as vitamin D dependency rickets type I (15, 19, 20). Follow-up studies have identified response elements within the 5'-flanking region of the rat CYP1α gene that allow it to be up-regulated by PTH, but in these same studies researchers were unable to identify elements responsible for down-regulation of the gene by $1\alpha,25(OH)_2D_3$ (21). The recent availability of the renal CYP1α sequence now allows for the first direct molecular examination of CYP1α in extrarenal tissues. In the present report, we provide evidence that the extrarenal CYP1α is a product of the same gene as the renal form and is detectable *in vitro* at both the messenger RNA (mRNA) and enzyme activity levels in certain colon and lung cancer cell lines that we studied. We speculate on the possible physiological role and importance of extrarenal CYP1α in the pathological processes resulting in the hypercalcemia of cancer.

Materials and Methods

Identification of an extrarenal 1α-hydroxylase-expressed sequence tag (EST)

The nucleic acid sequence from the mouse 1α-hydroxylase (GenBank accession no. AB006034) was used to search the human EST database for homologous sequences. Two human extrarenal complementary DNAs (cDNAs) were found. EST 587798 (1.84 kb) from a Stratagene (La Jolla, CA) colon library and EST 768387 (1.64 kb) from a Stratagene pancreas library. Both were obtained and sequenced. EST 587798 was identical to the corresponding portion of the human renal 1α-hydroxylase (GenBank accession no. AB005038), whereas EST 768387 was also identical, except that it contained a portion of genomic 1α-hydroxylase, similar to GenBank accession no. AB005990.

Nonsmall cell lung carcinoma (NSCLC) cell lines

SK-MES-1, SW 900, Calu-1, SK-Luci-6, and WT-E were provided by Dr. Barbara Campling. Cells were grown in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 5% FBS, penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37 C in an atmosphere of 5% CO_2 and 95% air.

Northern blot analysis

Poly(A)$^+$ RNA was isolated from cultured cells using the Oligotex Direct mRNA kit (QIAGEN, Valencia, CA) and electrophoresed on a formaldehyde-agarose gel. The gel was photographed under UV light and then blotted onto Hybond ECL nitrocellulose membrane (Amersham, Arlington Heights, IL) and fixed to the membrane by baking at 80 C for 2 h. Prehybridization and hybridization were performed using QuikHyb (Stratagene). A 1590-bp restriction fragment from EST 587798 was labeled with dATP[α-^{32}P] using the Prime-It II kit (Stratagene). The blot was washed twice for 15 min in 2× SSC (standard saline citrate)-0.1% SDS at room temperature, then for 15 min at 60 C in 0.1 × SSC-0.1% SDS and exposed at −70 C for 19 h to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY).

The same blot was stripped and reprobed in a similar manner using a labeled 408-bp PCR product generated from a human CYP24 cDNA clone (22) donated by Dr. H.F. DeLuca, University of Wisconsin-Madison.

RT-PCR analysis of cDNA

Total RNA was isolated from NSCLC cultured cells using TRIzol reagent (Life Technologies) followed by deoxyribonuclease I (Life Technologies) treatment. RT was performed on 2 μg total RNA using an AMV reverse transcriptase kit according to the manufacturer's protocol (Promega Corp., Madison, WI). PCR amplification was performed by mixing the following components (final concentrations) on ice: 0.2 mM dNTPs, 1 mM $MgCl_2$, 1 × PCR buffer (QIAGEN), 0.5 pmol/μl upstream primer, 0.5 pmol/μl downstream primer, 5 μl RT reaction, water to 49.5 μl, then 0.5 μl *Taq* DNA polymerase (5 U/μl; QIAGEN) was added. Reaction conditions were 1 cycle at 94 C for 2 min, followed by 35 cycles of 94 C for 30 sec, 58 C for 40 sec, and 72 C for 1.5 min, followed by a final extension of 5 min at 72 C. The oligonucleotides for extrarenal 1α-hydroxylase detection were based on the human renal 1α-hydroxylase sequence (17). The upstream primer was 5'-ACCATGACCCAGACCCTCAAGTA-3', and the downstream oligonucleotide was 5'-CTCTGAGCAAATGCAAACATCTGG-3'. The upstream and downstream oligonucleotides for CYP24 detection were 5'-AAACTAATGAAACCAGGGGAAGTG-3' and 5'-TCTGCACTAGGCTGCTGAGAATAC-3', respectively. To control for contamination of PCR samples due to CYP24 or CYP1α cDNAs, independent control reactions were set up in which no RT-generated cDNA was included. Ethidium bromide-stained gels showed an absence of products when we did not add specific cDNA samples. This was verified on the Southern analysis, which showed no hybridizing bands in the control lanes.

Southern analysis of RT-PCR products

The RT-PCR products were electrophoresed and blotted onto Nitro-Pure nitrocellulose transfer membrane (Micron Separations, Inc., Westboro, MA). Hybridization was performed at 42 C using gene-specific internal oligonucleotides, 5'-CTGCAGCTCGTGTAGCCTCGAC-3' for CYP1α and 5'-AAACGTGCTCATCATTGTTTTGAT-3' for CYP24. Oligonucleotides were end labeled using ATP[γ-^{32}P] and T4 polynucleotide kinase. The blots were washed and exposed at −70 C to Kodak X-Omat AR film (Eastman Kodak Co.).

Induction of CYP24 activity by 25OHD$_3$

SW 900 cells were cultured in RPMI 1640 supplemented with 5% FCS. Approximately 8.5 × 10^6 cells were washed with PBS twice, and the medium was replaced with RPMI 1640 supplemented with 1% BSA. The inducer 10 μM $25OHD_3$ and the antioxidant 100 μM 1,2-dianilinoethane were added, and the cells were incubated for 24 h. Total RNA was prepared using TRIzol reagent (Life Technologies), treated with deoxyribonuclease I, and reversed transcribed using an AMV reverse transcriptase kit according to the supplier's directions (Promega Corp.). PCR amplification was performed in a Perkin Elmer PCR machine as follows: 10 μl cDNA synthesis reaction, 2.5 U *Taq* DNA polymerase (Qiagen), 200 μM cDNA reaction dNTPs (contributed by the first strand cDNA reaction), 2 mM $MgCl_2$, 1 × RT buffer (Promega Corp.), and 1 μM of each upstream and downstream primer for CYP24 or CYP1α. PCR conditions were as follows: 1 cycle at 94 C for 2 min; 35 cycles at 94 C for 30 s, 56 C for 40 s, and 72 C for 1 min; followed by a final extension for 5 min at 72 C. Aliquots (10 μl) of the PCR reactions were electrophoresed and photographed. PCR reactions were controlled as previously described for RT-PCR analysis of cDNA.

Cloning of the full-length 1α-hydroxylase cDNA

The full-length PCR product from SW 900 was obtained using the upstream primer 5'-GGCGGATCCAGGGGTTGAGATATGATGCTCAGG-3' and the downstream primer 5'-GACGAATTCTGGTCAGATAGGCATTAGGGGAAG-3' according to the method outlined previously for RT-PCR. The product was purified using the QIAquick PCR purification kit (QIAGEN) and digested with *Eco*RI and *Bam*HI. The gene was then ligated into the pcDNA3.1(+) vector using T4 DNA ligase (Life Technologies), electroporated into competent *Escherichia coli*, plated on Luria Bertoni-ampicillin plates, and incubated overnight at 37 C. Colonies were grown up in Luria Bertoni-ampicillin medium, and DNA was prepared using High Pure Plasmid Isolation Kit (Boehringer Mannheim, Laval, Canada).

In vitro studies of vitamin D metabolism in cultured cells

Cells were grown to confluence in RPMI 1640 supplemented with 5% FCS on 100-mm plates, washed with PBS twice before the start of metabolic studies to minimize the sequestrating effect of vitamin D-binding

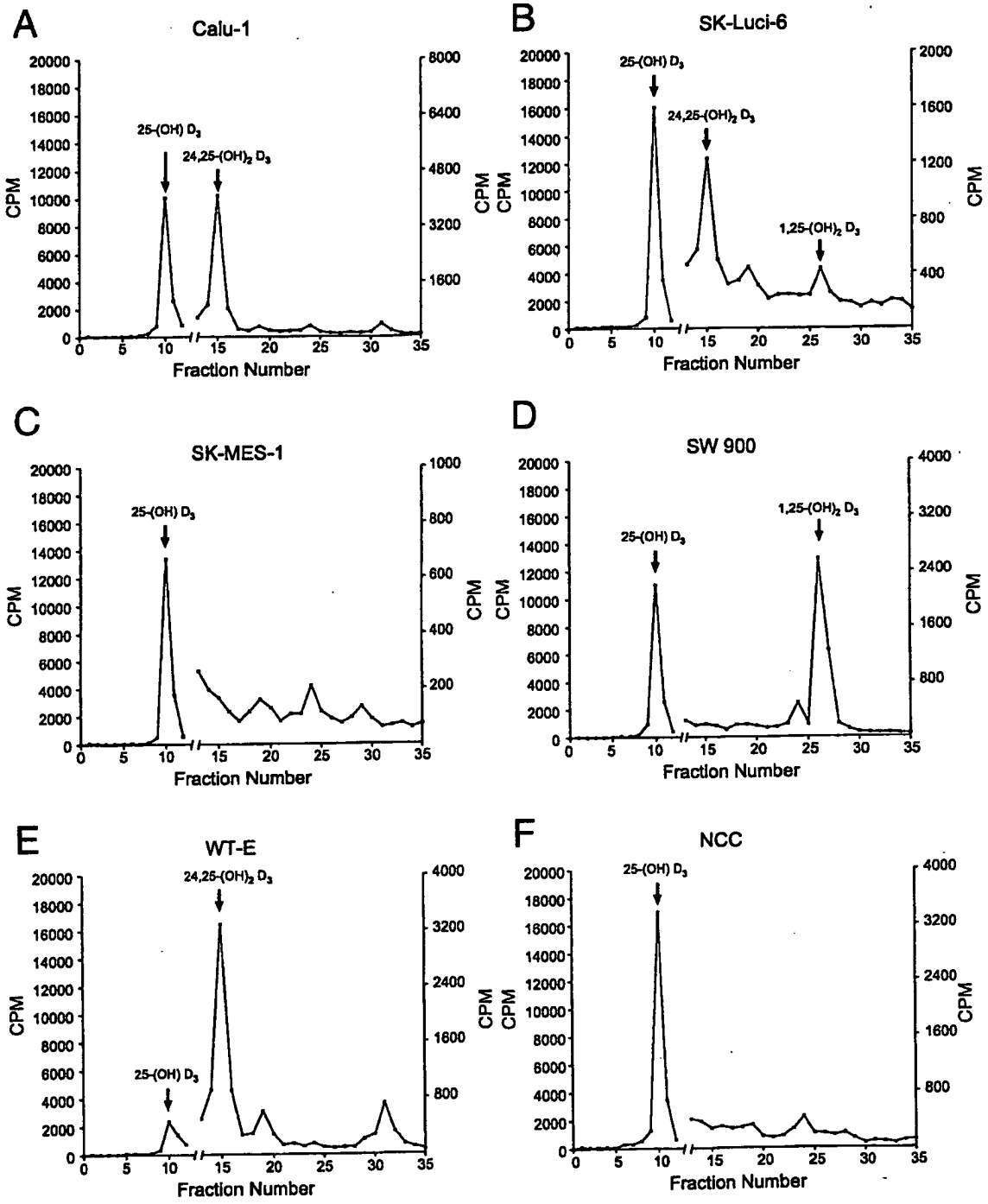

FIG. 1. HPLC of extract of five NSCLC cell lines incubated with [26,27-^{3}H]25OHD$_3$ for 24 h. Condition: Zorbax-SIL, 3 μm, hexane-2-propanol-methanol, 91:7:2, 1 ml/min. The effluent was collected in 30-sec aliquots. The retention times of standard 25OHD$_3$, 24,25-(OH)$_2$D$_3$, and 1,25-(OH)$_2$D$_3$ were 4.67, 7.11, and 12.44 min, respectively. A, Calu-1 cell. B, SK-Luci-6 cell. C, SK-MES-1 cell. D, SW 900 cell. E, WT-E cell. F, No cell control.

globulin, present in the FCS, on 25OHD$_3$ uptake by cells. Medium was replaced with RPMI 1640 supplemented with 1% BSA to act as a carrier of vitamin D and 100 μM 1,2-dianilinoethane, an antioxidant. Approximately 100,000 dpm [26,27-^{3}H]25OHD$_3$ (Amersham; SA, 20 Ci/mmol) was premixed with medium containing the above additives, then added to each plate of cells (4 ml/plate), and incubated for 24 h at 37 C in a 5% CO_2 atmosphere. Negative controls contained medium and radioactive substrate without cells. At the end of the incubation period, methanol was added to stop the enzymatic reaction and start the Bligh and Dyer lipid extraction procedure as described previously (23). Sample preparation for HPLC involved solubilization of the N_2-dried extract in 115 μl HPLC mobile phase, hexane-isopropanol-methanol in either 91:7:2 or 94:5:1 (vol/vol/vol).

In later experiments, confluent SW-900 cells were pretreated with 10

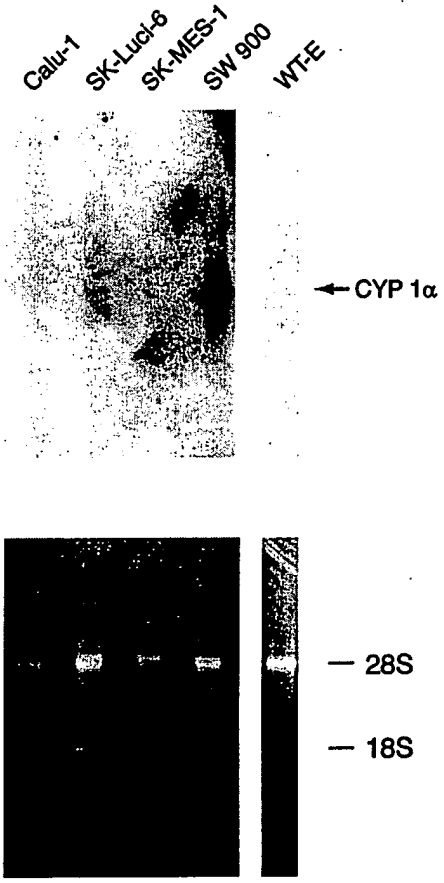

FIG. 2. Northern blot analysis of five NSCLC probed with a human CYP1α cDNA. The cell line SW 900 shows an abundant hybridizing transcript, as indicated by the *arrow*. Additionally, SK-Luci-6 cells show a faint signal for the CYP1α. Panels at the *bottom* show ethidium bromide-stained formaldehyde/agarose gels with the ribosomal RNA 18S and 28S locations indicated.

μM $25OHD_3$ to induce CYP24, and the cells were then washed with PBS to remove inducer, cultured with 70,000 dpm [26,27-^{3}H]$25OHD_3$, extracted, and prepared for HPLC as described above.

Analysis of vitamin D metabolites by HPLC

Straight phase LC was performed using a 2690 Alliance system (Waters Corp., Milford, MA) equipped with a Zorbax-SIL column and a diode array spectrophotometric detector recording in the 200–350 nm range. Solvent systems were mixtures of the solvents hexane-isopropanol-methanol in either 91:7:2 or 94:5:1 (vol/vol/vol; see figure legends for specific mixtures). Effluent was collected in 30-sec aliquots using a programmable fraction collector (Superrrac, Pharmacia, Montreal, Canada), and radioactivity in dried fractions was measured using a scintillation counter (Beckman Coulter, Inc., Palo Alto, CA).

In some experiments involving comigration of radioactive and nonradioactive $1,25-(OH)_2D_3$ standards, a Berthold radioflow detector (model LB509, Wallac Inc., Turku, Finland) was used. In other experiments where it was necessary to elute $1\alpha,24,25-(OH)_3D_3$, a linear straight phase gradient system was used from 91:7:2 to 88:10:2 hexane-isopropanol-methanol over 15 min starting at 0 min, followed by a hold at 88:10:2 hexane-isopropanol-methanol for 5 min before a reverse to starting conditions.

Reverse phase LC was performed using a 2690 Alliance system (Waters Corp.) equipped with a Zorbax-ODS column and a diode array spectrophotometric detector recording in the 200–350 nm range. A reverse phase gradient system was used from 50:50 to 100:0 acetonitrile-water over 25 min starting at 0 min, followed by a reverse to starting conditions. Effluent was collected in 15-sec aliquots using a programmable fraction collector (Superrrac, Pharmacia), and radioactivity in dried fractions was measured using an aqueous cocktail solution and a scintillation counter (Beckman Coulter, Inc.).

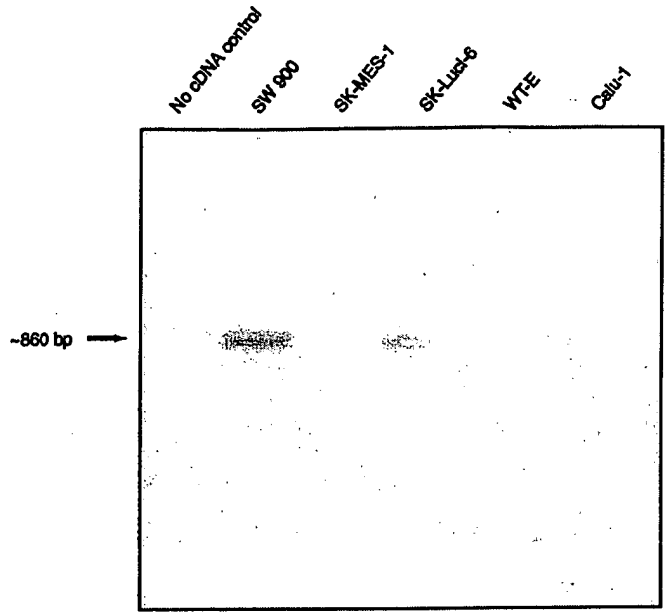

FIG. 3. Southern blot analysis of $25OHD_3$-1α hydroxylase (CYP1α) expression in five NSCLC. Two milligrams of total RNA were reverse transcribed and subjected to 35 cycles of PCR using gene-specific oligonucleotides as described in *Materials and Methods*. DNA was probed with a labeled internal oligonucleotide.

Transfection and enzyme activity of the full-length 1α-hydroxylase cDNA in COS-1 cells

COS-1 monkey kidney cells were subcultured 16–20 h before transfection and seeded in 100-mm plates at a density of 2×10^4 cells/cm^2. Transfection was carried out using the standard diethylaminoethyl-dextran procedure described in Guo *et al.* (24). Cells were transfected with the mammalian expression vector pcDNA3.1(+) containing full-length CYP1α, as constructed above, or the same pcDNA3.1(+) vector containing no insert. Approximately 24 h after transfection, cells were subcultured into six-well plates, and 24 h later 1α-hydroxylase enzyme activity was measured. For this, the transfected cells were washed twice with PBS, and 1 ml unsupplemented DMEM containing 1% BSA and 50,000 cpm [26,27-^{3}H]$25OHD_3$ was added to each plate. After a 6-h incubation, the reaction was stopped by the addition of methanol followed by extraction and chromatography as described above for lung cell cultures.

Results

Identification of a source of the extrarenal 1α-hydroxylase

By searching the human EST database at the National Center for Biotechnology Information, we identified a clone from the human colon cell line, T84, which showed homology to the previously characterized renal 1α-hydroxylase (17). Subsequent RT-PCR analyses have confirmed that this cell line contains transcripts for full-length CYP1α (data not shown). The nucleotide sequence of this partial clone was 100% identical over approximately two thirds of the published coding sequence of the human renal enzyme. As this cell line was derived from a putative secondary metastasis in

a patient with a suspected primary tumor of the lung, and as others had found 1α-hydroxylase enzyme activity in a lung cancer cell line (9), we focused upon screening other lung cancer cell lines for 1α-hydroxylase activity and CYP1α mRNA.

Correlation of 1α-hydroxylase enzyme activity and CYP1α mRNA expression

Screening of five NSCLC for 1α-hydroxylase enzyme activity revealed the results shown in Fig. 1. Two of the cell lines, SW 900 (Fig. 1D) and SK-Luci-6 (Fig. 1B), showed specific production of a putative peak of [26,27-^{3}H]1,25-$(OH)_2D_3$ on HPLC, which was absent in no cell control incubations (Fig. 1F). The peak of SW 900-generated [26,27-^{3}H]1,25-$(OH)_2D_3$ comigrated on straight phase HPLC using Zorbax-SIL and a radioactive monitor with both synthetic commercially available [26,27-^{3}H]1,25-$(OH)_2D_3$ (Amersham) and [1β-^{3}H]1,25-$(OH)_2D_3$, synthesized in our laboratory (25) [retention times: SW 900-generated [26,27-^{3}H]1,25-$(OH)_2D_3$, 12.167 min; synthetic [26,27-^{3}H]1,25-$(OH)_2D_3$, 12.179 min; synthetic [1β-^{3}H]1,25-$(OH)_2D_3$, 12.147 min]. Similarly, the SW 900-generated [26,27-^{3}H]1,25-$(OH)_2D_3$ and synthetic 1,25-$(OH)_2D_3$ comigrated exactly on reverse phase HPLC using Zorbax-ODS (retention time, 9.8 min). The peak was absent in no cell and dead cell controls, indicating that it is not an artifactual peak, such as 19-nor,10-keto-25OHD$_3$ reported previously in cell-free dilute protein solutions. In addition, the other cell lines, particularly WT-E (Fig. 1E) and Calu-1 (Fig. 1A), produced none of the [26,27-^{3}H]1,25-$(OH)_2D_3$, but, instead, produced significant quantities of the alternative metabolite 24,25-$(OH)_2D_3$ as well as small amounts of other side-chain oxidized products. When we used Northern analysis to examine cells for CYP1α mRNA, we again found positive signals of the correct size in only two of the five cells, with SW 900 showing the strongest signal. Upon longer exposure of the blot, a signal could be observed in SK-Luci-6 (Fig. 2). We have not probed the blots with controls for mRNA quantification; however, all samples were quantified by UV detection and verified visually on the gels before blotting. Northern data were corroborated by performing RT-PCR on the five cell lines; only SW 900 and SK-Luci-6 gave the expected 860-bp band that hybridized to a specific internal CYP1α oligonucleotide on Southern analysis (Fig. 3). Thus, there appears to be a correlation between 1α-hydroxylase enzyme activity and CYP1α mRNA expression in the five NSCLC lines.

Correlation of 24-hydroxylase enzyme activity and CYP24 mRNA expression

As we found clear-cut conversion of 25OHD$_3$ to 24,25-$(OH)_2D_3$ in two of the lung cell lines and because there appears to be a well established reciprocal relationship between the 1α-hydroxylase and 24-hydroxylase in the kidney, we chose to examine the cell lines for CYP24 activity and mRNA expression. On Northern analysis, the cell lines that showed high levels of CYP24 activity (WT-E and Calu-1) also gave strong positive signals for CYP24 mRNA (Fig. 4). In these cell lines, WT-E shows a single transcript as denoted by the *top arrow* (Fig. 4). We note that Calu-1 shows two signals,

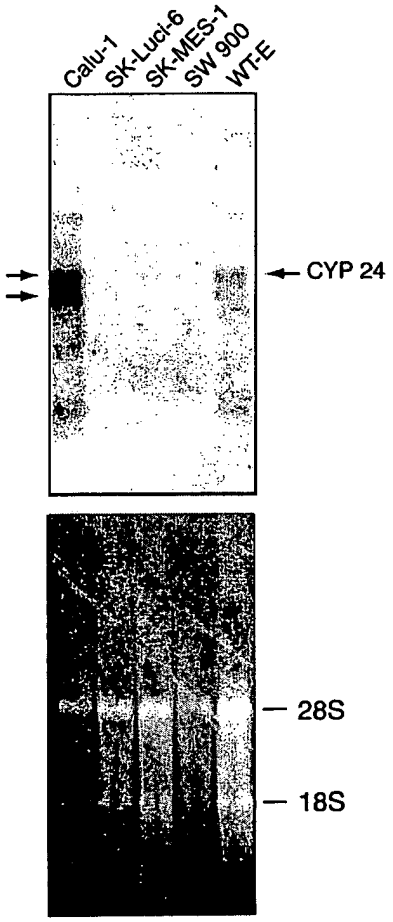

FIG. 4. Northern blot analysis of five NSCLC probed with a human CYP24 cDNA. The cell line Calu-1 shows two hybridizing transcripts, as indicated by the *arrows*. WT-E cells show a single transcript, also indicated by the *top arrow*. Panels at the *bottom* show ethidium bromide-stained formaldehyde/agarose gel, with the ribosomal RNA 18S and 28S locations indicated.

one of the same size as in WT-E and another slightly smaller. On RT-PCR, all cell lines were positive for CYP24 (data not shown). The discrepancy between the Northern analysis and RT-PCR is probably a reflection of the sensitivity of the PCR analysis. Thus, it appears that there is a correlation between the 24-hydroxylase enzyme activity measurements and Northern analysis for CYP24 mRNA, although there is a hint of 24-hydroxylase activity and/or CYP24 mRNA signal in SK-Luci-6 and SK-MES-1 cells.

Effect of pretreatment with vitamin D on enzyme activity and mRNA expression

The pretreatment of SW 900 cells with vitamin D was used to potentially induce CYP24 mRNA and 24-hydroxylase activity. As expected, 24-hydroxylase activity was induced, as the principal product generated from 25OHD$_3$ incubation was 24,25-$(OH)_2D_3$ (Fig. 5A), whereas vehicle-treated cells continued to produce 1,25-$(OH)_2D_3$ (Fig. 5B), and there were no products in the no cell controls (Fig. 5C). This result was confirmed by incubation of induced cells with 10 μM

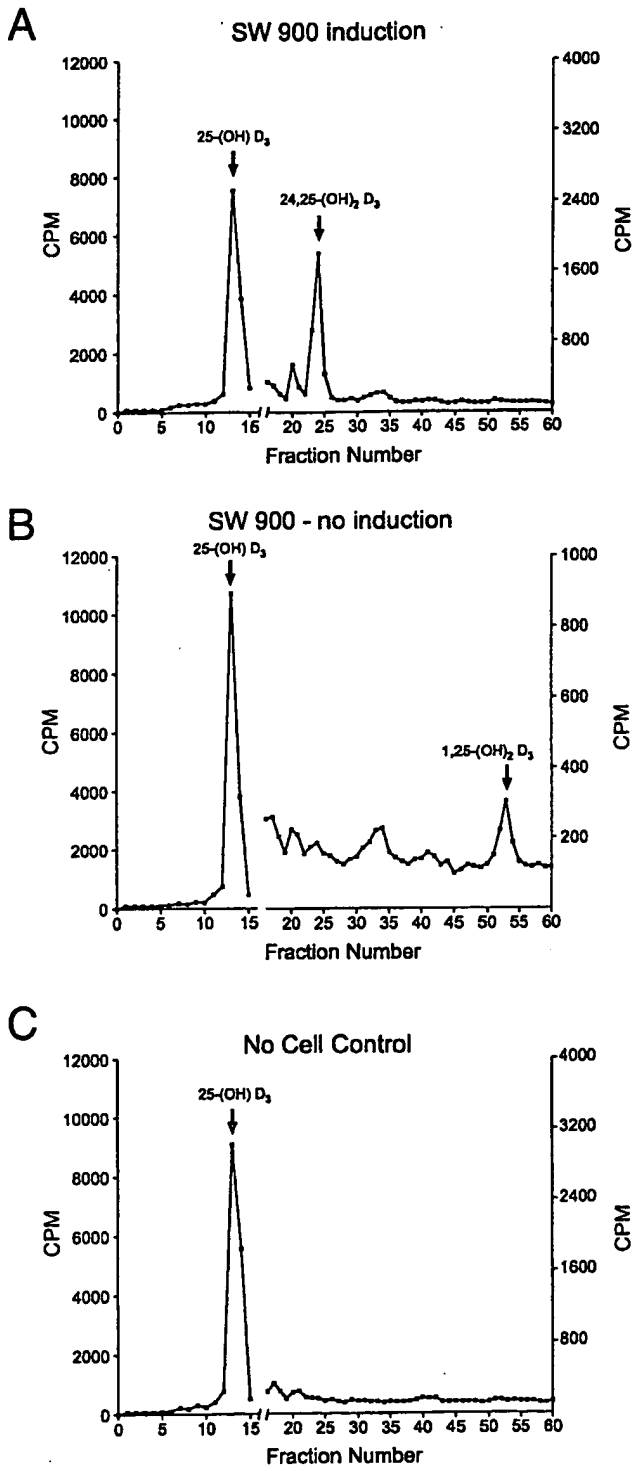

FIG. 5. HPLC of extract of SW 900 cells incubated with [26,27-^{3}H]25OHD$_3$ for 24 h. Condition: Zorbax-SIL, 3 μm, hexane-2-propanol-methanol, 94:5:1, 1 ml/min. The effluent was collected in 30-sec aliquots. The retention times of standard 25OHD$_3$, 24,25-(OH)$_2$D$_3$, and 1,25-(OH)$_2$D$_3$ were 6.26, 11.26, and 25.66 min, respectively. A, SW 900 cell treated with 10 μM 25OHD$_3$ for 24 h before incubation with [26,27-^{3}H]25OHD$_3$. B, SW 900 cell without pretreatment. C, No cell control.

25OHD$_3$ as substrate, which resulted in the formation of a detectable peak on HPLC with the retention time of standard 24,25-(OH)$_2$D$_3$ and the UV spectrum of a vitamin D (data not shown). The appearance of 24-hydroxylase enzyme activity in SW 900 cells was accompanied by the coincidental detection of CYP24 mRNA as a band of about 400 bp on RT-PCR (Fig. 6A), whereas in untreated or vehicle-treated cells no CYP24 band was evident, and only CYP1α was detectable as a band of around 860 bp. Interestingly, despite being induced to begin to express CYP24, SW 900 cells continued to express CYP1α mRNA 24 h after induction with vitamin D (Fig. 6B), although these cells failed to show production of the [26,27-^{3}H]1,25-(OH)$_2$D$_3$ metabolite. However, these extracts did contain a radioactive peak that comigrated with 1α,24,25-(OH)$_3$D$_3$ on gradient HPLC (data not shown), although it was not possible to tell whether this metabolite had been produced from [26,27-^{3}H]1,25-(OH)$_2$D$_3$.

Transfection studies with full-length 1α-hydroxylase

COS-1 cells transfected with the cDNA for the SW 900 1α-hydroxylase and incubated with [26,27-^{3}H]25OHD$_3$ produced a peak that comigrated with 1,25-(OH)$_2$D$_3$ on straight phase HPLC, whereas all transfection controls, including COS-1 cells transfected with empty vector, nontransfected COS-1 cells, and no cell controls, gave a flat baseline in the 1,25-(OH)$_2$D$_3$ region of the chromatogram (Fig. 7).

Discussion

We have described in this paper the molecular cloning of the putative extrarenal 25OHD$_3$-1α-hydroxylase from a human lung nonsmall cell carcinoma. It is clear that the expressed mRNA in this (and probably other) lung tumor cell(s) is the same size as that previously characterized in kidney and is the product of the same gene. As expected, the full-length SW 900 lung cell-CYP1α transfected into monkey kidney COS-1 cells gives rise to the same [26,27-^{3}H]1,25-(OH)$_2$D$_3$ metabolite peak formed by SW 900 cells under basal culture conditions. The cloning here of the human enzyme from lung and colon cancer cells together with the data of Fu *et al.* for an extrarenal 1α-hydroxylase of keratinocyte origin (18) suggests that there is only one form of the enzyme. It remains to be seen if the forms previously detected in alveolar macrophage and placenta are also identical.

In our studies of the five lung cell lines, we found that there was a good correlation of the expression of the mRNA for CYP1α (measured by Northern analysis or RT-PCR) and the 1α-hydroxylase enzyme activity or, alternatively, correlation of the mRNA for CYP24 (measured by Northern analysis) and the 24-hydroxylase enzyme activity. This reciprocal relationship between the two enzyme activities has been observed before, particularly for renal preparations of vitamin D-deficient and vitamin D-replete animals (26), but the P450 sequences now make it possible to confirm this at the mRNA level.

The findings of CYP1α expression in two lung cell lines, CYP24 expression in two others, and not much activity in the fifth cell line indicate inherent differences between the cell lines. It should be noted that all five small cell carcinomas were cultured under identical conditions. Thus, basal ionic,

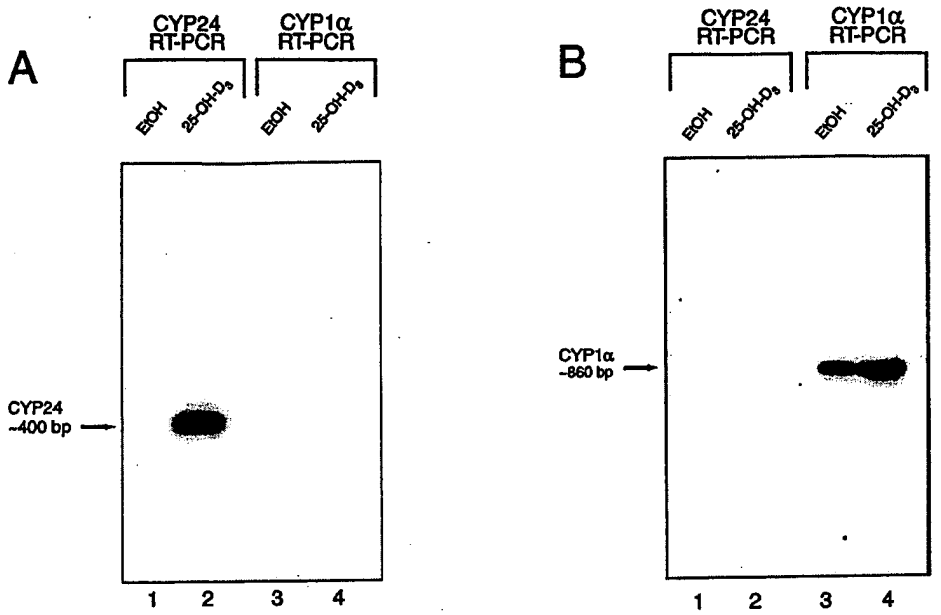

FIG. 6. RT-PCR Southern blot analysis of $25OHD_3$-24-hydroxylase (CYP24) and $25OHD_3$-1α hydroxylase (CYP1α) expression in the NSCLC cell line SW900. Cells were treated with ethanol vehicle (lanes 1 and 3) or $25OHD_3$ (lanes 2 and 4). The PCR products were then analyzed by gel electrophoresis and Southern blotted using a CYP24 gene-specific oligonucleotide (A). The Southern blot from A was stripped and rehybridized with a CYP1α gene-specific oligonucleotide (B).

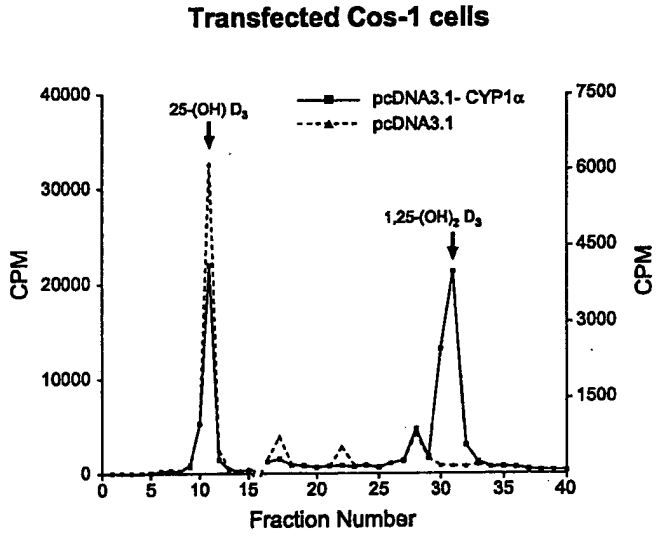

FIG. 7. HPLC of extracts of cells transfected with the mammalian expression vector pcDNA3.1(+) containing no insert (a; *dashed line*) or full-length CYP1α (b; *solid line*) and incubated with [26,27-^{3}H]$25OHD_3$ for 6 h. Condition: Zorbax-SIL, 3 μm, hexane-2-propanol-methanol, 91:7:2, 1 ml/min. The effluent was collected in 30-sec aliquots. The retention times of standard $25OHD_3$ and $1,25\text{-}(OH)_2D_3$ were 5.06 and 14.79 min, respectively.

vitamin D, or other hormone conditions should be identical, these being known factors in stimulating 1α-hydroxylase or 24-hydroxylase expression. However, the abilities of cell lines to produce and secrete autocrine or paracrine factors, such as PTH-related peptide (PTHrP) or cytokines (*e.g.* interferon-γ), probably differs between the different cell lines, and these may play a role in CYP expression (27). Other possible mechanisms modulating cytochrome P450 expression may include growth factor or peptide hormone receptor expression.

Our experiments have revealed that under basal conditions, the SW 900 cell line expresses CYP1α mRNA and shows 1α-hydroxylase enzyme activity, whereas in the same cells treated with exogenous vitamin D metabolites we observed expression of CYP24 mRNA and the synthesis of 24-hydroxylated metabolites. This switchover is similar to the process observed in the kidney (26). Thus, vitamin D seems to be a stronger and more overwhelming effector than the presumed unknown modulator that pushes the SW 900 cell line into constitutive CYP1α expression. Interestingly, RT-PCR using probes for CYP1α continues to show a signal of the correct size for CYP1α expression 24 h after the treatment with vitamin D and long after CYP24 has been induced, but the production of $1\alpha,25\text{-}(OH)_2D_3$ on HPLC is no longer detectable. There may be several possible explanations for the apparently conflicting data including the selective further metabolism of $1\alpha,25\text{-}(OH)_2D_3$. In fact, experiments performed here demonstrate the formation of $1\alpha,24,25\text{-}(OH)_3D_3$ after CYP24 appears in SW900 cells, and it is attractive to speculate that it is formed from $1\alpha,25\text{-}(OH)_2D_3$. Certainly, $1\alpha,25\text{-}(OH)_3$ is known to be an excellent substrate for CYP24 (28) and, if formed, would probably be quickly and preferentially converted to C-24 oxidation products such as $1\alpha,24,25\text{-}(OH)_3D_3$. The interesting phenomenon is the continued expression of CYP1α mRNA 24 h after treatment despite the presence of the $1\alpha,25\text{-}(OH)_2D_3$ signal, which would be expected to turn off CYP1α expression based upon earlier *in vivo* findings (29). However, the results here and the recent report of no discernible effect of $1\alpha,25\text{-}(OH)_2D_3$ on basal or PTH-induced expression of a rat CYP1α promoter-driven reporter gene *in vitro* (21) suggest that the previously observed suppressive action of $1\alpha,25\text{-}(OH)_2D_3$ *in vivo* must be at a posttranscriptional level or is indirect and requires the presence of some other agent absent from our *in vitro* model.

Another important implication of our work is the role of extrarenal 1α-hydroxylase in the pathogenesis of the hypercalcemia of cancer. All cell lines studied here were obtained randomly from tumor banks and therefore presumably re-

flect the incidence of CYP1α expression in the general pool of such tumors. This incidence is surprisingly high compared with that observed by Mawer *et al.* (9) and implies that the extrarenal production of 1α,25-$(OH)_2D_3$ may be more common than is currently believed, and part of the reason that this has not been reported more frequently reflects the technical difficulty that existed for proving the presence and activity of CYP1α.

PTHrP production by the lung cell lines studied here is also unknown, but is currently under investigation. The complex interrelationship of 1α,25-$(OH)_2D_3$ and PTH/PTHrP, in which 1α,25-$(OH)_2D_3$ down regulates PTH/PTHrP synthesis and PTH/PTHrP up-regulates the CYP1α gene, suggests that a paracrine loop may exist in extrarenal tissues for the regulation of cell growth/differentiation, a hypothesis put forward previously (12, 30). The demonstration of cancer cell lines in which extrarenal 1α,25-$(OH)_2D_3$ production is constitutive but subject to weak regulation makes it possible to pursue the study of the molecular events underlying the role of the extrarenal 1α-hydroxylase in this possible feedback loop.

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Exhibit B

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Transcriptional Synergism between Vitamin D-responsive Elements in the Rat 25-Hydroxyvitamin D_3 24-Hydroxylase (*CYP24*) Promoter*

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David M. Kerry, Prem P. Dwivedi, Christopher N. Hahn‡, Howard A. Morris§, John L. Omdahl¶‖, and Brian K. May

From the Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5005, Australia and the ¶Department of Biochemistry, University of New Mexico, Albequerque, New Mexico 87131

Transcription of the *CYP24* gene is induced by 1,25-$(OH)_2D_3$ through a vitamin D receptor-dependent process. The functional activities of three possible vitamin D response elements (VDREs), located on the antisense strand of the rat *CYP24* promoter, were investigated by transient expression of native and mutant promoter constructs in COS-1, JTC-12, and ROS 17/2.8 cells. A putative VDRE with a half-site spacing of 6 base pairs at −249/−232 (VDRE-3) did not contribute to 1,25-$(OH)_2D_3$ induced expression in the native promoter, although activity has been reported when the element was fused to the heterologous thymidine kinase promoter. Two VDREs with half-site spacings of 3 base pairs at −150/−136 and −258/−244 (VDRE-1 and VDRE-2, respectively), showed transcriptional synergism in COS-1 cells when treated with 1,25-$(OH)_2D_3$ (10^{-7} to 10^{-11} M). The contribution of both VDREs was hormone-concentration dependent from 10^{-10} to 10^{-12} M, with VDRE-1 demonstrating greatest sensitivity to 1,25-$(OH)_2D_3$. Transactivation by VDRE-1 was always greater than VDRE-2, but the converse was observed for the binding of vitamin D receptor-retinoid X receptor complex by each VDRE in gel mobility shift assays. The synergy observed between VDRE-1 and VDRE-2 may have important implications in cellular responses to different circulating levels of 1,25-$(OH)_2D_3$.

The hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$ [1] or calcitriol) is a pleiotropic secosteroid that functions in the regulation of calcium homeostasis, cellular differentiation and proliferation, and immune function (1–5). Nuclear actions of 1,25-$(OH)_2D_3$ involve the transcriptional regulation of gene expression, which is mediated by the vitamin D receptor (VDR), a ligand-activated transcription factor that belongs to the nuclear receptor superfamily (1, 6–10). Activated VDR can bind as either a homodimer or a heterodimeric complex to a DNA sequence known as the vitamin D-responsive element (VDRE) present in the promoter of target genes (11–13). Heterodimers consisting of VDR and retinoid X receptor (RXR) are widely documented (12), although VDR heterodimeric complexes have also been demonstrated for both the retinoic acid receptor (14, 15) and the thyroid hormone receptor (16). Vitamin D-responsive elements generally display a binding motif that consists of two imperfect, direct repeat hexameric sequences (*i.e.* half-sites) that are separated by 3 bp or, more rarely, by 6 bp; these VDREs are referred to as DR-3 and DR-6, respectively (12).

Metabolic inactivation of 1,25-$(OH)_2D_3$ and conversion to water-soluble calcitroic acid occurs through the C-24 oxidation pathway. The initial step in this pathway involves the 24-hydroxylation of 1,25-$(OH)_2D_3$ by the mitochondrial enzyme 25-hydroxyvitamin D_3 24-hydroxylase (CYP24) (17, 18). Rats fed a normal diet express a low level of CYP24, predominantly in the kidney. However, the enzyme can be substantially induced in kidney and intestine (19–23) and various other cells (24–29) by 1,25-$(OH)_2D_3$ treatment. Up-regulation of CYP24 expression (19–23) increases the metabolic clearance of 1,25-$(OH)_2D_3$, and, thereby, feedback regulates the hormone's ambient and cellular levels (17, 18). The mechanism whereby 1,25-$(OH)_2D_3$ acts to modulate cellular CYP24 expression is of fundamental importance to understanding the hormone's role in health and disease.

Molecular regulatory studies of *CYP24* gene expression by 1,25-$(OH)_2D_3$ are in progress and promoter analysis data for rat (14, 30–33) and human (34) have been reported. In the rat *CYP24* gene promoter, three VDREs on the antisense strand have been identified. We have previously defined the proximal VDRE (30) in its native promoter context, while the two more distal VDREs have been tested by fusing to a heterologous promoter (14, 31). To date, however, the functionality of the VDREs has not been verified in the context of the native *CYP24* promoter, and there is no direct information available regarding the contribution of each VDRE to vitamin D induction or whether there is a cooperative interaction between the response elements. These issues are addressed in the current investigation, in which mutagenic constructs of the rat *CYP24* promoter have been used in transient gene expression and gel mobility shift analysis.

EXPERIMENTAL PROCEDURES

Materials—Hoffmann La Roche (Nutley, NJ) generously supplied the 1,25-$(OH)_2D_3$. A Sequenase version 2.0 sequencing kit was obtained from U. S. Biochemical Corp. Synthetic oligonucleotides were synthesized by Bresatec (Adelaide, Australia). Luciferin was from Promega Corp. (Madison, WI).

Construction of Mutant Clones—A 365-bp *PvuII/StuI* fragment containing 298 bp of 5′-flanking sequence and encompassing the putative VDREs together with 74 bp of 5′-untranslated region was isolated from a rat *CYP24* genomic clone (30) and used as template for site-directed

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‡ Current address: Dept. of Genetics, St. Jude Children's Research Hospital, Memphis, TN 38101.

§ Current address: Clinical Biochemistry, Institute of Medical and Veterinary Science, Adelaide, South Australia 5000, Australia.

‖ To whom correspondence should be addressed. Current address: Dept. of Biochemistry, University of New Mexico, Albuquerque, NM 87131. Tel.: 505-277-5791; Fax: 505-277-5389; E-mail: omdahl@medusa.unm.edu.

[1] The abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-$(OH)D_3$, 25-hydroxyvitamin D_3; CYP24, 25-hydroxyvitamin D_3 24-hydroxylase; VDR, vitamin D receptor; VDRE, vitamin D-responsive element; RXR, retinoid X receptor; DR-3/DR-6, direct repeat VDREs; mSpp1, mouse osteopontin; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

mutagenesis. The fragment was cloned into the *Hin*cII site of pBluescript KS$^+$ in the antisense orientation to generate pBKS-WT, and single-stranded DNA purified as described by Kunkel *et al.* (35). Briefly, *Escherichia coli* CJ236 (dut$^+$, ung$^-$) was transformed with pBKS-WT and superinfected with helper phage M13K07. Single-stranded DNA was purified by NaCl/PEG precipitation and used as template in the mutagenesis reactions. The primers employed (P1, P2, P4, and P5) contain mutations (shown as bold letters) in VDRE-1, VDRE-2, VDRE-2, and VDRE-3, and VDRE-3, respectively: P1, 5′-CGC CCT CAC TCA **AGC TTC** TGA CTC CAT CCT CTT CCC ACA CCC-3′; P2, 5′-GTG CTC GCA GCG **CAT** GCG CTG AAC CCT GGG CTC GAC CCG CCT-3′; P4, 5′-GTG CTC GCA GCG **CAT GCG CAC** TAC CCT GGG CTC GAC CCG CCT-3′; P5, 5′-GTG CTC GCA GCG CAC CCG CTG AAC CCT GGG CTA TAC CCG CCT-3′. The positions of these mutations are shown in Fig. 1. After annealing each mutant oligonucleotide to the template, the second DNA strand was completed using T4 DNA polymerase. Positive clones were isolated by colony screen hybridization using tetramethylammonium chloride washes (36). Mutant clones were verified by sequence and restriction enzyme analysis. In VDRE-1, a *Hin*dIII site was introduced to produce the mutated nucleotides.

Generation of Promoter Constructs—The *CYP24* mutant promoter fragments were excised from pBKS-WT by digestion with *Xho*I/*Eco*RV and cloned into *Xho*I/*Sma*I-digested pGL3-Basic containing the firefly luciferase reporter gene (Promega). Plasmid DNA was prepared by alkaline lysis and CsCl/ethidium bromide equilibrium density gradients (37). All plasmid DNA was quantified by spectrophometry, and supercoil form was estimated by 1% agarose gel analysis to ensure experimental consistency.

Maintenance and Transfection of Tissue Culture Cells—COS-1 cells and monkey kidney proximal tubular JTC-12 cells (kindly supplied by Dr T. Matsumoto, University of Tokyo School of Medicine, Japan; Ref. 38) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Rat osteosarcoma-derived ROS 17/2.8 cells were maintained in DMEM/Ham's F-12 (1:1) supplemented with 10% FCS. In preparation for electroporation, cells were grown to 80–90% confluence, removed by trypsinization, washed once in phosphate-buffered saline and resuspended at (6×10^6 cells/ml) in 20 mM Hepes (pH 7.05) containing 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose (39), and 500 μg/ml sheared salmon sperm DNA. COS-1 and JTC-12 cells (3×10^6) and 1 pmol of construct DNA were electroporated at 280 V and 960 microfarads, while ROS 17/2.8 cells were electroporated at 200 V and 960 microfarads using a Bio-Rad Gene Pulser. COS-1 cells were also co-transfected with VDR expression clone pRSV-hVDR, generated by cloning the human VDR cDNA sequence (kindly supplied by Nigel Morrison, Garvan Institute for Medical Research Sydney, Australia) downstream of the Rous sarcoma virus promoter. Following electroporation, the samples were placed on ice for 10 min and divided (1.5×10^6 cells) into two wells of a six-well plate containing DMEM, 10% FCS (for COS-1 and JTC-12 cells) or DMEM/Ham's-F-12, 10% FCS (for ROS 17/2.8 cells). Cells were allowed to recover for 20 h and then the media replaced with RPMI medium (without phenol red) supplemented with 12% charcoal-stripped FCS. Ethanol carrier or 1,25-$(OH)_2D_3$ was added at the indicated concentration and the cells incubated for 24 h prior to harvesting.

Luciferase Assay—Cells were washed once with phosphate-buffered saline and treated with 200 μl of Cell Culture Lysis Reagent (Promega) for 10 min at room temperature. The cells were harvested with a rubber policeman, frozen on dry ice, thawed on ice, and vortexed vigorously. After centrifugation, the supernatant was removed and assayed for protein concentration. Luciferase activity was determined using a luciferase assay system (Promega) in 25 μg of lysate protein and measurements made with a Berthold model LB 9502 Luminometer.

Gel Mobility Shift Assays—Double-stranded oligonucleotide probes were synthesized that encompassed the VDRE-1, VDRE-2, or VDRE-3 sites in the rat *CYP24* promoter. An oligonucleotide that encompassed a known VDRE located at −758/−740 in the mouse osteopontin gene promoter (40) was employed as control (mSpp1-VDRE). Each double-stranded oligonucleotide was designed with *Sal*I and *Xho*I overhangs at the 5′ or 3′ ends, as shown below.

VDRE-1: 5′-TCGAGCGGC**GCCCCT**CAC**TCACCT**CGCG-3′
3′-CGCC**GCGGGA**GTG**AGTGGA**GCGCAGCT-5′

VDRE-2: 5′-TCGACCAGC**GCACC**CGC**TGAACC**CTGC-3′
3′- GGTC**GCGTGG**GCG**ACTTGG**GACGAGCT-5′

VDRE-3: 5′-TCGACGC**TGAACC**CTGGGC**TCGACC**CGC-3′
3′-GCG**ACTTGG**GACCCG**AGCTGG**GCGAGCT-5′

mSpp1: 5′-TCGACGCTCGGGTAG**GGTTCA**CGA**GGTTCA**CTCGACTCGC-3′
3′-GCGAGCCCATC**CCAAGT**GCT**CCAAGT**GAGCTGAGCGAGCT-5′

SEQUENCES 1–4

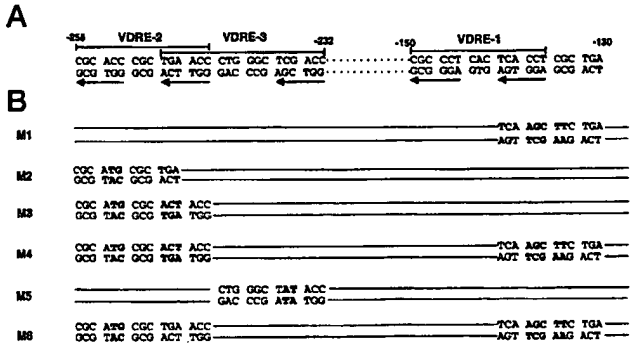
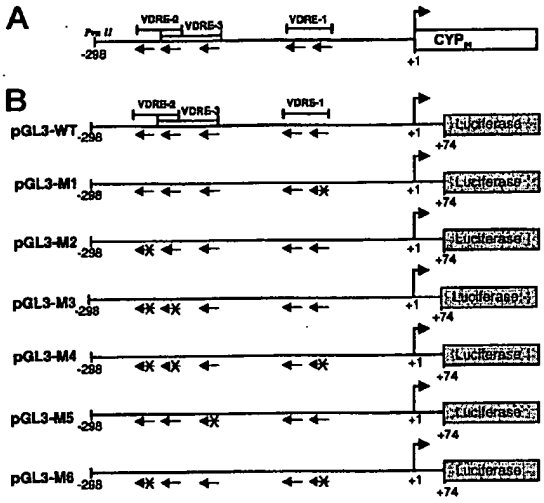


FIG. 1. **Mutations introduced into the VDREs located in the rat *CYP24* gene promoter.** *A*, sequence of the native promoter encompassing the three VDREs. The *numbers* above the *lines* indicate the distance relative to the transcription initiation site. *Arrows* indicate VDRE hexameric half-sites. *B*, different mutations (shown in *bold type*) introduced into VDRE-1 (*M1*), VDRE-2 (*M2*), VDRE-2 and VDRE-3 (*M3*), all three VDREs (*M4*), VDRE-3 (*M5*), and VDRE-1 and VDRE-2 (*M6*).

Each double-stranded oligonucleotide was labeled by end-filling with [α-^{32}P]dCTP using Klenow enzyme and purified by 12% polyacrylamide gel electrophoresis. Nuclear extracts from 1,25-$(OH)_2D_3$-treated COS-1 cells were prepared with or without co-transfection of pRSV-hVDR expression vector, as described previously (30). Binding reactions for each assay contained 5 μg of nuclear protein, 1 μg of poly(dI-dC) to a final volume of 12 μl in binding buffer (25 mM Tris-HCl, pH 7.6, 100 mM KCl, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM EDTA, and 10% glycerol) and were incubated on ice for 10 min. Radiolabeled probe (200,000 cpm) was added and samples incubated on ice for another 20 min. For gel shift inhibition assays, a VDR monoclonal antibody designated IgG2b (Affinity BioReagents Inc., Neshanic Station, NJ) was employed. For supershift assays, an RXR monoclonal antibody (4RX-1D12, Ref. 41), kindly provided by Dr Pierre Chambon (Strasbourg Cedex, France), was employed. These antibodies were included in the binding reactions and incubated on ice for 10 min prior to addition of probe. Gel shift competition assays were performed with unlabeled competitor oligonucleotide at molar excess concentrations in the binding reaction. Retarded DNA nuclear protein complexes were resolved on a 4% nondenaturing polyacrylamide gel in a low ionic strength running buffer (0.5 × TBE) at 4 °C. The gel was dried and exposed to Kodak X-Omat AR film with an intensifying screen at −70 °C.

RESULTS

Transient Expression of Promoter Constructs in COS-1, JTC-12, and ROS 17/2.8 Cells—Mutational analysis was used to determine the functionality and cooperative interaction of three putative VDREs in the rat *CYP24* gene promoter. The three VDREs (*i.e.* VDRE-1, VDRE-2, and VDRE-3) were altered, either individually or in combination, by site-directed mutagenesis (Figs. 1 and 2). The proximal half-site of VDRE-1



	COS-1	JTC-12	ROS 17/2.8
pGL3-WT	17.8 ± 1.8	14.3 ± 2.5	17.8 ± 3.8
pGL3-M1	2.8 ± 0.9	2.2 ± 0.4	1.7 ± 0.2
pGL3-M2	6.6 ± 1.8	5.6 ± 1.3	7.8 ± 3.4
pGL3-M3	6.4 ± 2.1	5.2 ± 1.2	6.1 ± 1.5
pGL3-M4	0.9 ± 0.3	0.9 ± 0.3	1.2 ± 0.3
pGL3-M5	18.4 ± 2.2	13.4 ± 1.9	17.3 ± 4.6
pGL3-M6	1.3 ± 0.3	ND	0.9 ± 0.2

FIG. 2. **Transient expression of wild-type and mutant *CYP24* promoter constructs in different mammalian cell lines.** *A*, diagrammatic representation of the promoter region of the rat *CYP24* gene. *B*, luciferase expression of pGL3-promoter luciferase constructs containing wild-type (*pGL3-WT*) and mutated VDREs (*pGL3-M1–pGL3-M6*) in the promoter. COS-1 cells only were co-transfected with 1 μg of pRSV-hVDR. The levels of induction are shown as the ratio of luciferase activity from 1,25-$(OH)_2D_3$-treated cells to that from untreated cells. Data presented are the average of three separate experiments ± S.D. *ND*, not determined. *Arrows* indicate VDRE hexameric half-sites, with *X* indicating a mutation site.

and the distal half-site of VDRE-2 were each mutated in the constructs pGL3-M1 and pGL3-M2, respectively. Both half-sites of VDRE-2 (which included a common half-site with VDRE-3) were mutated in construct pGL3-M3. Mutations were introduced into all three VDREs in construct pGL3-M4, while only VDRE-3 was mutated in construct pGL3-M5. In pGL3-M6, half-sites in both VDRE-1 and VDRE-2 were mutated, leaving VDRE-3 intact. The impact of the half-site mutations on 1,25-$(OH)_2D_3$-directed transcriptional activation of the gene promoter was evaluated in COS-1, JTC-12, and ROS 17/2.8 cells. In the absence of added 1,25-$(OH)_2D_3$, basal expression of each of the mutant constructs in the different cell lines was the same as the wild-type construct, demonstrating that these VDREs do not contribute to basal expression (results not shown).

Transient transfection experiments were performed initially in COS-1 cells that were co-transfected with *CYP24* promoter/luciferase constructs and VDR expression vector (pRSV-hVDR) to compensate for the deficiency of VDR in the COS-1 cell line. In response to 1,25-$(OH)_2D_3$ treatment, the wild-type construct (pGL3-WT) gave a 17.8-fold level of induction in these cells (Fig. 2). When VDRE-1 alone was mutated (pGL3-M1), leaving the distal VDRE-2 and VDRE-3 intact, the level of 1,25-$(OH)_2D_3$ induction was reduced to 2.8-fold, which demonstrated the substantial contribution of VDRE-1 to promoter activity. A 6.6-fold level of induction was observed with the pGL3-M2 construct in which VDRE-2 was mutated, and essentially the same level was observed when both VDRE-2 and VDRE-3 were mutated in pGL3-M3 (Fig. 2). These findings established that both VDRE-1 and VDRE-2 are functional and suggested that VDRE-3 does not significantly contribute to induction even when the overlapping VDRE-2 is inactivated. To confirm this latter finding, the expression of pGL3-M5, in which the proximal half-site in VDRE-3 was altered, was investigated. The hormone induction observed with this construct was similar to the wild type. The possible role of VDRE-3 was examined further by mutating both VDRE-1 and VDRE-2 and leaving VDRE-3 intact. This construct (pGL3-M6) was inactive (Fig. 2). There was no response to 1,25-$(OH)_2D_3$ by the construct pGL3-M4, in which all three VDREs were altered. Thus, when evaluated in all three cell types, VDRE-1 and VDRE-2 were responsible for the 1,25-$(OH)_2D_3$-mediated induction of the wild-type promoter region. The induction seen for the wild-type construct (17.8-fold) was greater than the sum of the individual contributions of VDRE-1 (6.6-fold) and VDRE-2 (2.8-fold), thus demonstrating transcriptional synergism.

Cell specificity for the synergism between VDRE-1 and VDRE-2 was also evaluated in JTC-12 and ROS 17/2.8 cells. Both cell lines express endogenous VDR and respond to 1,25-$(OH)_2D_3$-mediated gene induction (38, 42). Expression of the wild-type and mutant *CYP24* promoter constructs in JTC-12 and ROS 17/2.8 cells produced transactivation results that were similar to those obtained in COS-1 cells (Fig. 2). A nearly 2-fold synergistic action between VDRE-1 and VDRE-2 was demonstrated in all three cell lines, in which a greater contribution was observed for VDRE-1 compared with VDRE-2 (Fig. 2).

VDREs Bind Nuclear Protein Complexes—Binding of nuclear proteins to the VDREs was investigated by gel mobility shift analysis using extracts from COS-1 cells transfected with pRSV-hVDR expression vector and treated with 10^{-7} M 1,25-$(OH)_2D_3$ (30). An oligonucleotide encompassing the mouse osteopontin VDRE (*i.e.* mSpp1-VDRE) was employed as a control probe and contained a functionally active VDRE known to bind strongly the VDR-RXR complex (40). A major protein complex of the same mobility as the mSpp1-VDRE was detected with VDRE-1 and VDRE-2, but the bands were not evident when probes were incubated with nuclear extract prepared from COS-1 cells not transfected with pRSV-hVDR (Fig. 3*A*). This finding indicated that VDR is present in the protein complex that binds to VDRE-1 and VDRE-2. Competition experiments using a 10-fold molar excess of mSpp1-VDRE completely prevented formation of the protein complex observed with either VDRE-1 or VDRE-2 (results not shown). To further characterize the protein complex, we employed both a monoclonal antibody to VDR that interfered with DNA binding of VDR and a supershifting RXR monoclonal antibody. The VDR monoclonal antibody prevented formation of the major protein complex detected when radiolabeled VDRE-1, VDRE-2, or mSpp1-VDRE were used as probes (Fig. 3*B*). The RXR monoclonal antibody supershifted the VDR-containing complex obtained with each of these probes (Fig. 3*C*). It can be concluded from these data that the major protein complex that binds to VDRE-1 and VDRE-2 contains both VDR and RXR. Other gel mobility shift experiments with mutant oligonucleotides established that the mutations introduced into VDRE-1 and VDRE-2 inhibited completely the binding of the VDR·RXR complex (data not shown).

Based upon the intensity of the protein-VDRE complexes, it appeared that VDRE-2 had a greater binding affinity than

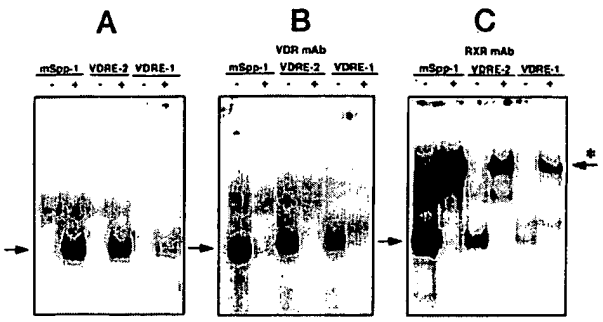


FIG. 3. **Gel shift analysis using oligomers to VDRE-1 and VDRE-2.** *A*, double-stranded oligomers to VDRE-1, VDRE-2, and mouse osteopontin VDRE (*mSpp1-VDRE*) were labeled by end-filling with [α-^{32}P]dCTP and incubated with nuclear extracts of COS-1 cells either mock-transfected (−) or transfected with 5 μg of pRSV-hVDR (+). The major retarded complex (*arrowed*) is unique to pRSV-hVDR-transfected cells. *B*, for neutralization assays, nuclear extracts (from COS-1 cells transfected with pRSV-hVDR) were incubated with (+) or without (−) VDR monoclonal antibody (*VDR mAb*) prior to addition of labeled probes for VDRE-1, VDRE-2, and mSpp1-VDRE. *C*, for supershift assays, as in *B* but using RXR monoclonal antibody (*RXR mAb*). The *upper arrow* (*) indicates the supershifted complex.

VDRE-1 while the mSpp1-VDRE had a higher binding affinity than either VDRE-1 or VDRE-2 (Fig. 3). To investigate the binding affinity of VDRE-1 and VDRE-2 more directly, competition of the radiolabeled mSpp1-VDRE probe was undertaken with either unlabeled VDRE-1 or VDRE-2 at 10-, 25-, 50-, 100-, and 200-fold molar excess. The results showed that the protein complex was efficiently competed by a 10-fold excess of the competitor VDRE-2, but a 50-fold excess of VDRE-1 was required for significant competition (Fig. 4*A*). These results confirmed that the complex containing VDR and RXR has a stronger binding affinity for VDRE-2 than VDRE-1. Competition experiments were also carried out using radiolabeled VDRE-1 or VDRE-2 as probes and each of these oligonucleotides as competitors. With self-competition, binding of the VDR·RXR protein complex to VDRE-1 and VDRE-2 was totally abolished with the corresponding oligonucleotides, confirming the specificity of protein binding (Fig. 4*B*). With VDRE-1 as probe, a 10-fold molar excess of VDRE-2 effectively prevented protein complex formation, while in the reverse experiment, a 50-fold molar excess of VDRE-1 was required. These experiments further demonstrated the greater binding affinity of VDRE-2.

Using COS-1 nuclear extracts, we have detected a very faint retarded protein complex with the VDRE-3 probe in gel shift assays and antibody experiments (data not shown), which demonstrated that this complex contained VDR and RXR. The biological significance of VDR·RXR binding to VDRE-3 is not clear in view of the inactivity of the sequence in the *CYP24* promoter. In other studies, DR-6 type VDREs, and in particular VDRE-3, have been reported to preferentially bind VDR homodimers and retinoic acid receptor-VDR heterodimers rather than VDR·RXR (12, 14).

Studies in COS-1 Cells Treated with Different Amounts of 1,25-(OH)$_2$D$_3$ or VDR—Having used experimental conditions in which 1,25-(OH)$_2$D$_3$ was non-limiting (10^{-7} M), it was decided to evaluate promoter activity in COS-1 cells under more physiological conditions by incrementally decreasing the level of hormone challenge to 10^{-12} M. The maximal level of promoter induction seen with 10^{-7} M 1,25(OH)$_2$D$_3$ (about 18-fold; Fig. 2) was retained with concentrations of 1,25-(OH)$_2$D$_3$ as low as 10^{-10} M (Fig. 5*A*). However, when cells were treated with 10^{-11} M 1,25-(OH)$_2$D$_3$, the induction was reduced to 8.3-fold and was further lowered to 3.7-fold with 1,25-(OH)$_2$D$_3$ at 10^{-12} M (Fig. 5*A*). Mutant constructs of VDRE-1 (pGL3-M1) and VDRE-2 (pGL3-M2) were active at 10^{-11} M 1,25-(OH)$_2$D$_3$, giving 2.1-fold and 3.3-fold levels of induction, and they retained their transcriptional synergism. At the lowest level of 1,25-(OH)$_2$D$_3$ tested (10^{-12} M), only VDRE-1 was found to be responsive (Fig. 5*A*). Separate experiments confirmed that VDRE-3 did not contribute to the induction response (when VDRE-1 and VDRE-2 were functional) under the decreasing concentrations of 1,25-(OH)$_2$D$_3$ employed (data not shown).

The possible impact of cellular VDR concentration on the synergistic interaction between VDRE-1 and VDRE-2 was evaluated in COS-1 cells receiving one-tenth the amount of the transfected pRSV-hVDR. Under these conditions, the wild-type promoter construct gave an 8.0-fold level of induction compared with 16.4-fold when cells were co-transfected with the full amount of pRSV-hVDR (Fig. 5*B*). Studies with the mutant constructs pGL3-M1 and pGL3-M2 also showed a decreased level of induction, but the cooperative interaction between VDRE-1 and VDRE-2 was retained. Upon comparing results at different 1,25-(OH)$_2$D$_3$ and VDR levels, it was noted that VDRE-1 makes a greater contribution to the hormone's cellular induction than VDRE-2. Such results are diametric to the VDR·RXR binding affinities for the two VDREs, as measured by gel mobility shift analysis.

DISCUSSION

Transcription of the rat cytochrome P450$_{24}$ (*CYP24*) gene is induced by 1,25-(OH)$_2$D$_3$ (19–23). Three VDREs in the first 298 bp of the 5′-flanking sequence have been reported to participate in regulation of the rat *CYP24* gene (14, 30–33). To date, only the proximal VDRE (VDRE-1) has been evaluated for function in the context of its native promoter (30). The other putative VDREs (VDRE-2 and VDRE-3) were characterized when linked to the heterologous thymidine kinase promoter (14, 31), which does not reflect native promoter architecture and function. Using constructs containing the first 298 bp of the *CYP24* gene promoter, we have now characterized VDRE-1 and VDRE-2 as functional hormone response elements. Up-regulation of the *CYP24* promoter regulation in three different cell lines is consistent with the operation of a general modulatory loop that functions in 1,25-(OH)$_2$D$_3$-responsive cells expressing the *CYP24* gene. A very similar regulatory pathway also appears to be present in the human *CYP24* promoter, which has been verified to contain two VDREs of the DR-3 type at about the same position as the VDRE-1 and VDRE-2 in the rat (34).

VDRE-3 with a 6-bp spacing was not responsive to 1,25-(OH)$_2$D$_3$ when tested in three cell lines following selected mutation of VDRE half-sites. The data from these cellular promoter expression studies demonstrate convincingly that VDRE-3 is not functional. Our findings with VDRE-3 are in contrast to those of Kahlen and Carlberg (14), who reported that this sequence responds to 1,25-(OH)$_2$D$_3$ (4–6-fold) when fused to the thymidine kinase promoter and expressed transiently in ROS 17/2.8 cells. We conclude that the environment of the VDRE-3 in the rat *CYP24* promoter is not conducive to 1,25-(OH)$_2$D$_3$ responsiveness, at least in the cell types examined, and our study emphasizes the importance of investigating the functional role of a VDRE in the context of its native promoter architecture. Nevertheless, it is possible that VDRE-3 may be active in an appropriate cellular environment, since the sequence has been shown in gel mobility shift assays to bind VDR homodimers, heterodimers of VDR, and retinoic acid receptors (14) and, in the present work, to bind weakly a complex of VDR·RXR (data not shown). In the human *CYP24* promoter (34), the sequence 5′-ATGCGAacgccgGAGTTC-3′ corresponds to the rat VDRE-3, but its function has not been investigated. Other DR-6 type VDREs have been identified in

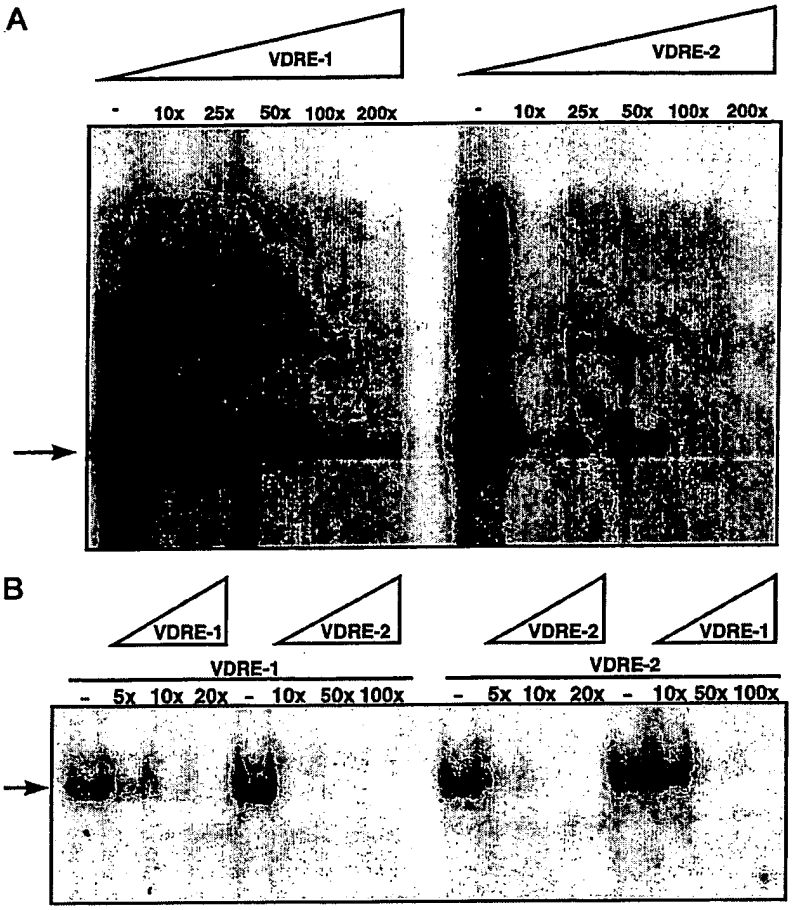


FIG. 4. **Competition studies with VDRE-1 and VDRE-2.** *A*, a double-stranded oligomer to mSpp1-VDRE was labeled by end-filling with [α-^{32}P]dCTP, incubated with nuclear extracts from COS-1 cells transfected with 5 μg of pRSV-hVDR, and competed with unlabeled VDRE-1 or VDRE-2 at 10-, 25-, 50-, 100-, or 200-fold molar excess. The major protein complex containing VDR·RXR is *arrowed*. The reduced intensity of bands in the *10× lane* for VDRE-2 is due to a loading variation. *B*, double-stranded oligomers for VDRE-1 and VDRE-2 were labeled by end-filling, incubated with nuclear extracts as in *A*, and competed with either VDRE at 5-, 10-, and 20-fold molar excess for self-competition and 10-, 50- and 100-fold excess for the cross-competition. The VDR·RXR complex is *arrowed*.

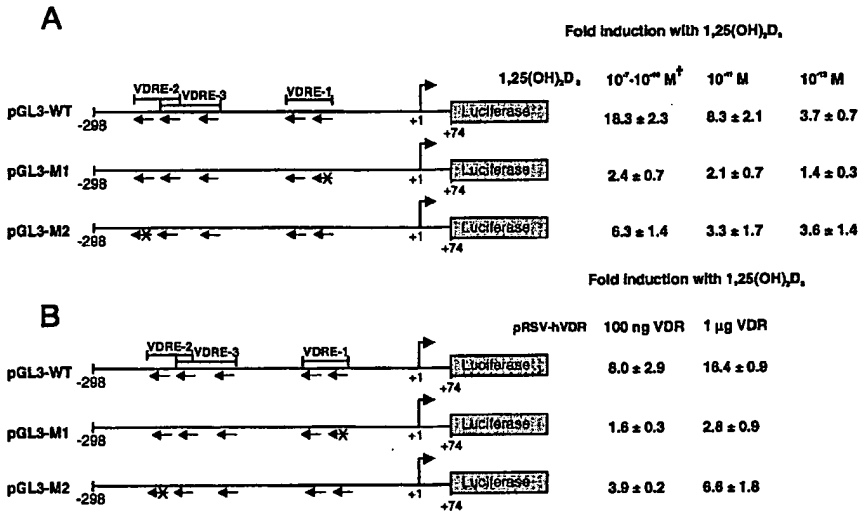


FIG. 5. **Transient expression of promoter-luciferase constructs in COS-1 cells in the presence of different concentrations of 1,25-$(OH)_2D_3$ and pRSV-hVDR.** *A*, induction of luciferase expression in COS-1 cells treated with 1,25-$(OH)_2D_3$ at a concentration range of 10^{-12} to 10^{-7} M and co-transfected with 1 μg of pRSV-hVDR. Values shown (†) are for 10^{-10} M 1,25$(OH)_2D_3$; essentially identical values were seen with 10^{-7}, 10^{-8}, and 10^{-9} M. The levels of luciferase expression are shown as the ratio of luciferase activity from 1,25-$(OH)_2D_3$-treated cells to that from untreated cells. Data presented are the average of three experiments ± S.D. *B*, -fold induction of luciferase activity in COS-1 cells co-transfected with 100 ng or 1 μg of pRSV-hVDR and treated with 10^{-7} M 1,25-$(OH)_2D_3$. *Arrows* indicate VDRE hexameric half-sites, with *X* indicating a mutation site.

the promoters for osteocalcin and fibronectin (12). The human osteocalcin DR-6 motif confers 1,25-$(OH)_2D_3$ inducibility when fused to the thymidine kinase promoter (13). However, as far as we are aware, it has not been established whether the osteocalcin DR-6 or fibronectin DR-6 motifs contribute to 1,25-$(OH)_2D_3$ responsiveness in their natural promoters.

Gel mobility shift analysis was used to investigate the protein complexes in COS-1 cell nuclear extracts that bind to VDRE-1 and VDRE-2. A major retarded band was identified with each VDRE, and antibody studies established that this complex contained both VDR and RXR. This result agrees with other studies, where different DR-3 type VDRE sequences have been shown to bind this heterodimer (12). Gel shift competition data indicated that the VDR·RXR protein complex bound more tightly to VDRE-2 than VDRE-1, a somewhat unexpected result, since the contribution of VDRE-1 to the 1,25-$(OH)_2D_3$ inductive response was always greater than that of VDRE-2. Liu and Freedman (43) have demonstrated substantial tran-

scriptional synergism between VDR and various classes of nonreceptor transcription factors when the DNA binding sites for these proteins are positioned closely in a reporter plasmid. Such a situation may explain the greater transactivation of VDRE-1, in which its affinity for VDR·RXR (compared with VDRE-2) is enhanced through a cooperative interaction with nearby, and as yet unidentified, promoter-bound transcription factor(s). It is also possible that the low 1,25-$(OH)_2D_3$ transactivation contributed by VDRE-2 arises from the greater distance of this VDRE from the transcriptional machinery. This seems unlikely, however, since deleting the promoter region between the two VDREs does not alter transactivation by 1,25-$(OH)_2D_3$ (31).

A significant finding in the present work is the transcriptional synergistic response between VDRE-1 and VDRE-2, which resulted in an 18-fold level of induction in COS-1 cells. Transient studies of *CYP24* promoter constructs in COS-1 cells under non-limiting conditions for VDR or 1,25-$(OH)_2D_3$ showed that the transactivation was about twice the sum of the hormone-dependent transactivations for VDRE-1 and VDRE-2 when evaluated separately. A similar level of synergism between these VDREs was also seen in JTC-12 and ROS 17/2.8 cells in which VDR is expressed endogenously. The mutations introduced into VDRE-1 or VDRE-2 inhibited completely the response of each site to 1,25-$(OH)_2D_3$ induction. In previous work (30), mutated VDRE-1 prevented 1,25-$(OH)_2D_3$ induction from a *CYP24* promoter that contained this VDRE but not VDRE-2. Combined mutagenesis of VDRE-1 and VDRE-2, in the current work, prevented induction and demonstrated the effectiveness of both mutations. Hence, the coordinated interaction between the two VDREs appears to be the sole basis for the observed synergism.

Synergistic induction of the rat *CYP24* promoter constitutes one of the highest stimulations observed so far for any 1,25-$(OH)_2D_3$-responsive promoter and would appear to be the only regulatory region in which two VDREs have been shown to be functional in their native promoter. The synergism observed between the two VDREs may be at the level of enhanced cooperative DNA binding of the VDR·RXR protein complexes. Liu and Freedman (43) have demonstrated cooperative binding of dimeric VDR complexes to two VDRE sites separated by 50 bp in an artificial promoter. However, Zierold *et al.* (31) were unable to show cooperative binding between protein complexes bound to sequences encompassing VDRE-1 and VDRE-2 in gel shift experiments. Another possible mechanism could involve independent binding of the protein complexes to each VDRE, with synergism resulting from an interaction of these complexes with the basal transcription machinery (43, 44). Of possible relevance to this latter mechanism is the demonstration that VDR can interact with the general transcription factor TFIIB (45).

Maximal induction of the *CYP24* promoter in COS-1 cells was observed over a 1000-fold range of 1,25-$(OH)_2D_3$ concentration (*i.e.* 10^{-7} M to 10^{-10} M). Therefore, it would appear that the VDREs can function maximally in a synergistic manner at physiological concentrations of 1,25-$(OH)_2D_3$. Continued decline in hormone concentration to 10^{-11} M resulted in VDRE-1 being the dominant response element, although synergism was retained between the two VDREs. The same effect was observed with similar 1,25-$(OH)_2D_3$ concentrations in ROS 17/2.8 cells, but a 10-fold higher concentration of hormone was required in JTC-12 cells (results not shown). The reason for the reduced sensitivity in these cells is under investigation. Nevertheless, the collective results establish synergism occurrence over a physiological range of 1,25-$(OH)_2D_3$ concentration in which VDRE-1 is always the major contributor to induction, particularly at low hormone concentrations.

The transcriptional synergism between the two VDREs in the promoter of *CYP24* may have important biological consequences. The induction of CYP24 by 1,25-$(OH)_2D_3$ constitutes an interesting feedback mechanism whereby 1,25-$(OH)_2D_3$ acts to regulate its metabolic clearance rate and, thereby, influence its ambient concentration. Increased levels of CYP24 activity will result in elevated side-chain oxidation of 1,25-$(OH)_2D_3$ and ultimately conversion to the water-soluble calcitroic acid and subsequent excretion (17, 18). In the normal situation, CYP24 is expressed predominantly in the kidney but can be induced by 1,25-$(OH)_2D_3$ in this tissue, in the intestine (19–23), and also in a variety of other cell types (24–29). It seems probable, therefore, that CYP24 expression in different tissues not only protects the tissue from the effects of excessive 1,25-$(OH)_2D_3$ but also regulates serum hormone levels by increasing its metabolic clearance rate. A synergistic response would ensure rapid removal of hormone when levels are sufficiently high to cause hypercalcemia and accelerated bone resorption (46). It can be predicted that VDRE-1 is preferentially utilized at lower 1,25-$(OH)_2D_3$ levels, but at higher hormone levels both VDREs are activated and transcriptional synergism facilitates the efficient inactivation of 1,25-$(OH)_2D_3$.

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